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The Mitochondrial Receptor Complex: A Central Role of MOM22 in Mediating Preprotein Transfer from Receptors to the General Insertion Pore

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Summary

The receptor complex in the mitochondrial outer membrane, which consists of at least seven different proteins, is responsible for the recognition and translocation of cytosolically synthesized preproteins. Two of its subunits, MOM19 and MOM72, function as surface receptors for preproteins. Four other subunits (MOM38, MOM30, MOM8, and MOM7) have been suggested to constitute the general insertion pore (GIP). Here we report on the structure and function of MOM22. MOM22 is anchored in the outer membrane by a single transmembrane segment. The highly negatively charged N-terminal domain is exposed to the cytosol and the C-terminal domain to the intermembrane space. MOM22 appears to be a central component of the receptor complex, required for the transfer of preproteins from the receptors to the GIP. We speculate that the negatively charged domain of MOM22 is involved in the transfer of positively charged signal sequences of preproteins.

Introduction

Translocation of preproteins across intracellular membranes is a process of considerable complexity involving a series of steps such as recognition, unfolding, membrane insertion, membrane passage, processing, and folding of the proteins (Wickner and Lodish, 1985; Neupert et al., 1990; Baker and Schatz, 1991; Keegstra and von Heijne, 1992; Rapoport, 1992; Sanders and Schekman, 1992). It is therefore not unexpected that not only a large number of components are required for preprotein translocation, but that these components are also organized in multisubunit complexes (Alberts and Miake-Lye, 1992).

Previous studies on the import of preproteins into mitochondria led to the identification of a protein complex in the mitochondrial outer membrane that mediates the specific recognition, membrane insertion, and translocation of preproteins across the outer membrane (Kiebler et al., 1990;

Moczko et al., 1992; Söllner et al., 1992). Seven different proteins, termed mitochondrial outer membrane proteins (MOMs), were found in this receptor complex. Two components of the complex were identified as receptors for preproteins. The 19 kd protein MOM19 seems to be the main receptor, involved in the import of most preproteins analyzed (Söllner et al., 1989, 1992; Steger et al., 1990; Pfanner et al., 1990; Schneider et al., 1991; Becker et al., 1992; Lill et al., 1992). In particular, MOM19 was found to be required for the import of preproteins with N-terminal signal sequences (presequences) as well as for the import of several noncleavable preproteins. The 72 kd protein MOM72 preferentially functions as receptor for a class of noncleavable preproteins with internal signal sequences, such as the precursors of the inner membrane proteins ADP/ATP carrier (AAC) and phosphate carrier (Söllner et al., 1990, 1992; Steger et al., 1990). In addition, both receptors possess overlapping specificities, allowing the import of MOM72-dependent precursors via MOM19 and vice versa, albeit with reduced efficiency (Steger et al., 1990; Hines et al., 1990; Söllner et al., 1992; Hines and Schatz, 1993). The two import receptors have a similar topology in that they are anchored in the outer membrane by a hydrophobic N-terminal segment, while the rest of the molecule is hydrophilic and exposed to the cytosol, where it is easily degraded by added proteases (Hase et al., 1984; Steger et al., 1990; Schneider et al., 1991).

Four other components of the receptor complex, MOM38 (also termed ISP42), MOM30, MOM8, and MOM7, have a different topology and function (Vestweber et al., 1989; Baker et al., 1990; Kiebler et al., 1990; Moczko et al., 1992; Söllner et al., 1992). They are in proximity of precursors arrested in the general insertion pore (GIP) and assumed to contribute to formation of the pore, which facilitates the insertion and translocation of almost all different preproteins (Pfanner and Neupert, 1987; Pfaller et al., 1988; Kiebler et al., 1990; Söllner et al., 1992). These four components as well as preproteins accumulated at the GIP are not degraded by proteases added to isolated mitochondria. The protease treatment leaves the GIP site intact as it does not affect the further transport of the accumulated preproteins to the inner membrane (Pfanner and Neupert, 1987; Pfanner et al., 1987b; Pfaller et al., 1988). Preproteins accumulated at the GIP expose portions to the intermembrane space, indicating that the GIP mediates the translocation of preproteins across the outer membrane (Rassow and Pfanner, 1991).

What then is the function of MOM22, the seventh component of the receptor complex? Since MOM22 is known to be exposed on the surface of the outer membrane (Kiebler et al., 1990), it may be expected that it has a function in an early step of translocation. One might speculate that a component is needed for the transfer of preproteins from the surface-exposed receptors MOM19 and MOM72 to the membrane-embedded components of the GIP. We thus investigated the structure and function of MOM22. MOM22 has domains both in the cytosol and in the inter-

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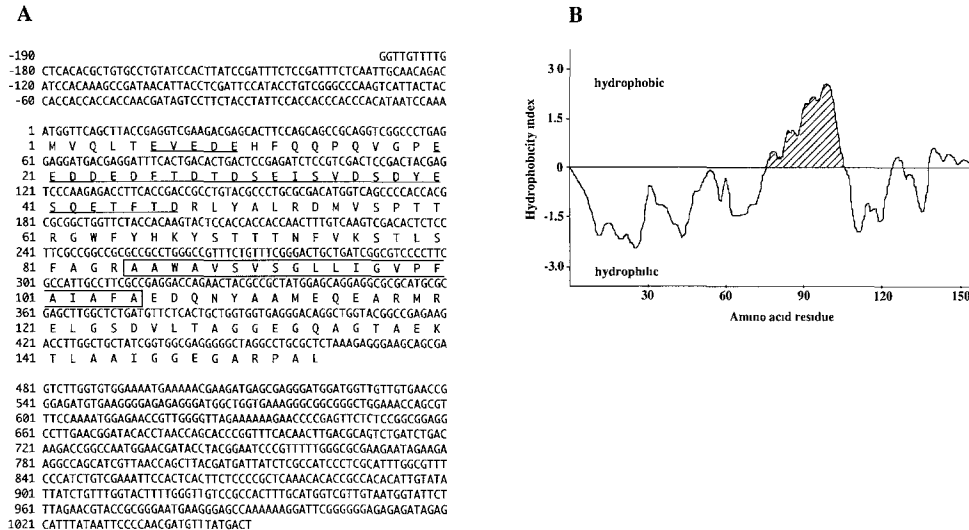


Figure 1. cDNA Sequence and Derived Amino Acid Sequence of MOM22

(A) Nucleotide sequence of the cDNA coding for *N. crassa* MOM22 and derived amino acid sequence (single letter code). The box indicates the putative membrane-spanning segment (residues 85–105). A cluster of negatively charged amino acid residues in the N-terminal third is underlined. (B) Hydropathy plot according to Kyte and Doolittle (1982). The putative membrane-spanning segment is hatched.

membrane space. Binding of antibodies to the cytosolic domain of MOM22 did not inhibit the interaction of precursors with the receptors MOM19 and MOM72, but blocked the passage of the precursors to the GIP, suggesting that MOM22 acts at a level between receptors and the GIP. We propose that the negatively charged cytosolic domain of MOM22 is needed for facilitating the membrane insertion of positively charged signal sequences.

Results

Deduced Primary Sequence of MOM22

As a tool for the identification of MOM22, an antiserum was available that reacted with both *Neurospora crassa* MOM19 and MOM22 (Kiebler et al., 1990). We screened an *N. crassa* cDNA library with the antiserum and obtained two groups of clones. One group encoded MOM19 (Schneider et al., 1991); the other group contained an open reading frame coding for a protein of 154 amino acid residues (16.8 kd) (Figure 1A). As shown below, antibodies directed against peptides of the N-terminus or the C-terminus of the deduced sequence selectively recognized MOM22 of the mitochondrial receptor complex and thus identified the cDNA as that for MOM22. The predicted molecular mass of MOM22 is smaller than that of MOM19. Depending on the gel systems used, MOM22 was found to migrate faster or slower than MOM19 (Moczko et al., 1992; see below). The hydropathy plot (Figure 1B) indicated the presence of two hydrophilic portions that are separated by a 21 residue uncharged hydrophobic sequence (residues 85–105) (Figure 1A). The N-terminal region is characterized by an exceptional prevalence of negatively charged residues (18 negative charges and 0 positive charges from residues 6–47) (Figure 1A). A search

of the EMBL/GenBank data bases did not reveal a significant homology of MOM22 to any protein on record.

MOM22 Is an Integral Outer Membrane Protein with an Unusual Cluster of Negative Charges in the N-Terminal Domain

To obtain monospecific antibodies against MOM22, peptides corresponding to residues 1–12 and 142–154 of the predicted sequence were synthesized and used to raise antisera in rabbits. Both antibodies, termed anti-MOM22N and anti-MOM22C, specifically recognized a single band among total mitochondrial proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2A, lanes 4 and 5). This band was also labeled by the original anti-MOM19/MOM22 antiserum (Figure 2A, lane 2), but not by an anti-MOM19 antibody (lane 1), identifying it as MOM22. The MOM22 precursor synthesized in vitro in rabbit reticulocyte lysate (Figure 2A, lane 6) had the same apparent size as the mature protein. This was to be expected, as all MOMs identified so far do not have cleavable presequences (Hartl et al., 1989; Baker and Schatz, 1991).

The receptor complex was immunoprecipitated from digitonin-lysed ³⁵S-labeled mitochondria with anti-MOM19 antibodies (Kiebler et al., 1990). The immunoprecipitate was then dissociated, and a second immunoprecipitation with specific anti-MOM22 serum was performed under stringent conditions (Figure 2B). MOM22 was thereby selectively precipitated, confirming that it is part of the receptor complex. Similarly, when the receptor complex purified in chemical amounts (Moczko et al., 1992) was immunodecorated with anti-MOM22 antibodies, a selective labeling of MOM22 was found (data not shown).

Thin sections of *N. crassa* cells were decorated with anti-MOM22 and gold-conjugated anti-rabbit antibodies.

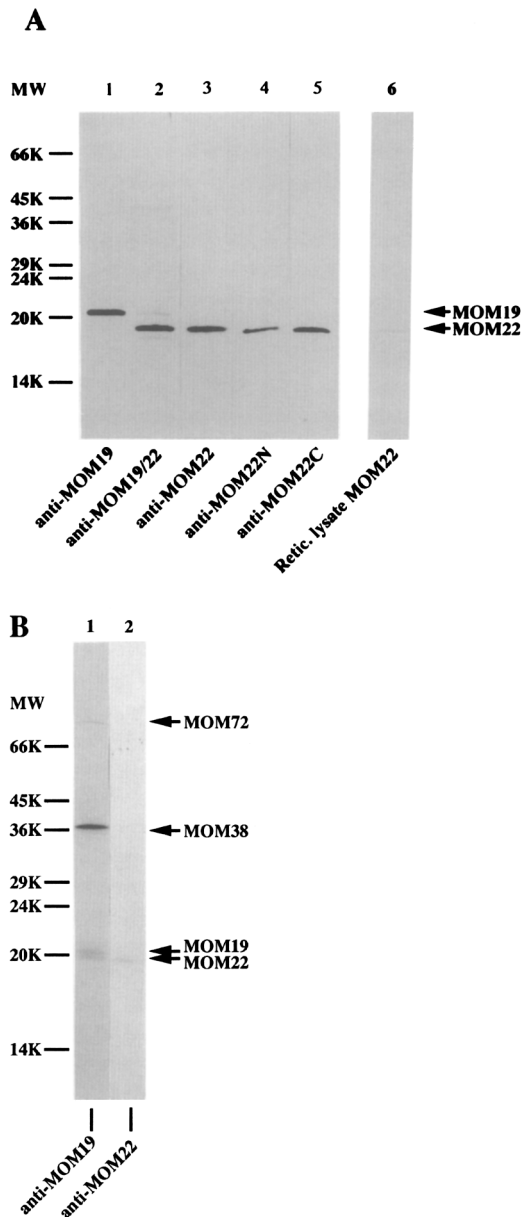


Figure 2. Identification of MOM22 as a Component of the Mitochondrial Receptor Complex by Monospecific Antibodies

(A) Identification of MOM22. *N. crassa* mitochondrial proteins (50 µg per lane) were resolved by SDS-PAGE, electrotransferred to nitrocellulose, and immunodecorated with various antisera (using the ECL Detection System, Amersham): lane 1, anti-MOM19 (Söllner et al., 1989); lane 2, anti-MOM19/MOM22 (Kiebler et al., 1990); lane 3, anti-MOM22 that was prepared against a fusion protein between MOM22 (amino acids 1–84) and the maltose-binding protein (see Experimental Procedures); lane 4, anti-MOM22N (directed against the N-terminus of MOM22); lane 5, anti-MOM22C (directed against the C-terminus of MOM22). In lane 6, MOM22 cDNA was transcribed and translated in reticulocyte lysate in the presence of [³⁵S]methionine, and then SDS-PAGE, electroblotting, and autoradiography were performed.

(B) Presence of MOM22 in the mitochondrial receptor complex. ³⁵S-labeled mitochondria (50 µg of protein) were solubilized with 0.5% digitonin and subjected to immunoprecipitation with anti-MOM19 antibodies prebound to protein A-Sepharose. The immunoprecipitate was dissociated in SDS-containing buffer. One half of the sample was directly subjected to SDS-PAGE (lane 1). The remaining half was diluted 40-fold in 1% (v/v) Triton X-100-SEM buffer, subjected to immunopre-

cipitation (Pfanner and Neupert, 1985) with anti-MOM22 antibodies, and further analyzed as described above (lane 2). The immunoprecipitation of the receptor complex was performed under the originally described conditions (Kiebler et al., 1990), which led to a coprecipitation, particularly of the "core" components MOM19, MOM22, MOM38, and a fraction of MOM72 (Moczko et al., 1992), suggesting that MOM22 is relatively stably associated with MOM19 and MOM38.

The gold particles were found at the periphery of the mitochondria (Figures 3A and 3B). Furthermore, isolated mitochondria were labeled with anti-MOM22 and gold-conjugated anti-rabbit antibodies prior to embedding and sectioning. This led to decoration of the outer membrane (Figure 3C). A similar result was obtained when anti-MOM22N antibodies were used (Figure 3D). Anti-MOM22C antibodies, however, did not bind to intact mitochondria; occasionally, we saw a labeling of vesicles (Figure 3E) that may represent inverted outer membranes derived from a few nonintact mitochondria in the preparation. The immunocytochemical results thus suggest that the N-terminus of MOM22 is exposed on the mitochondrial surface and that the C-terminus is located on the inter-membrane space side.

The uncharged hydrophobic sequence in the middle of MOM22 is of sufficient length to act as a possible membrane anchor. We tested whether MOM22 was an integral membrane protein by treating mitochondria at alkaline pH (pH 11.5). By this procedure, soluble and peripheral membrane proteins are extracted, while integral membrane proteins remain in the membrane sheets (Fujiki et al., 1982). MOM22 was indeed fully resistant to an extraction at alkaline pH (Figure 4A), suggesting that it is an integral membrane protein. Furthermore, MOM22 remained associated with the membranes upon sonication and salt treatment (Figure 4B).

The components of the mitochondrial receptor complex studied so far can be grouped into two classes according to their protease accessibility. The receptors MOM19 and MOM72 are easily degraded by proteases added to intact mitochondria (Söllner et al., 1989, 1990), while the components of the GIP, such as MOM38, are quite protected against proteases (Kiebler et al., 1990; Söllner et al., 1992). In Figure 4C we analyzed the sensitivity of MOM22 in intact mitochondria to various proteases (proteinase K, trypsin, and elastase) and found an intermediate behavior between that of the receptors and MOM38. Treatment with each of the proteases led to degradation of MOM22 to a fragment with an apparent size of 12 kd (Figure 4C) that was stable at even high concentrations of protease, unless the outer membrane was opened by detergent prior to the protease treatment (see below). At lower concentrations of proteinase K and elastase, intermediate-sized fragments of 19 kd and 16 kd were observed (Figure 4C).

The topology of MOM22 suggested by the electron microscopy data was confirmed by demonstrating that the 12 kd fragment lacked the N-terminal part. The fragment was recognized by the anti-MOM22C antibody, but not the anti-MOM22N antibody (Figure 4D). It was digested by protease after lysing mitochondria with Triton X-100 or

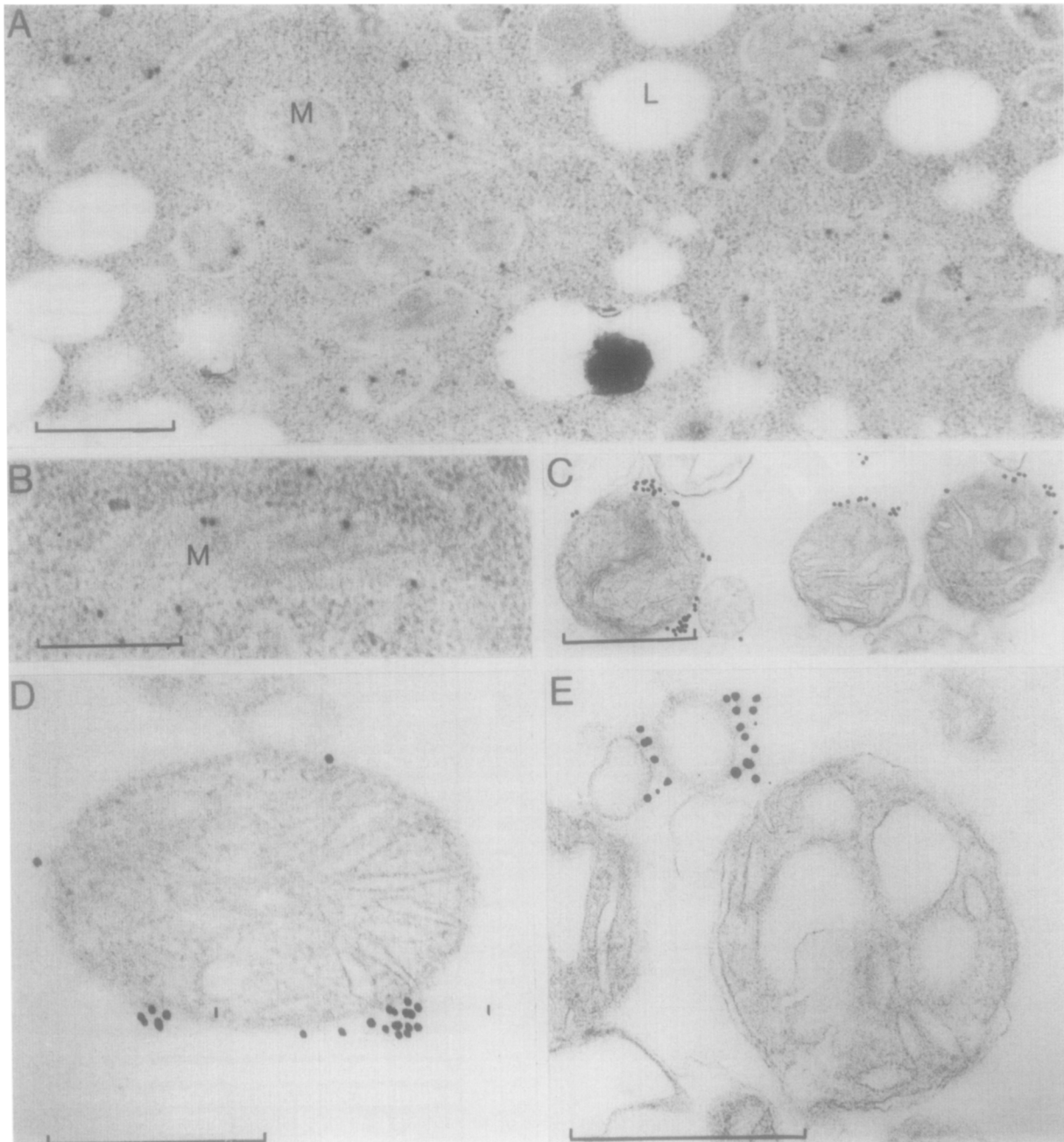


Figure 3. Immunocytochemical Localization of MOM22 to the Mitochondrial Outer Membrane

(A and B) Immunolabeling of ultrathin sections of Lowicryl-embedded *N. crassa* cells. The sections were incubated with anti-MOM22 antibodies and gold-conjugated goat anti-rabbit antibodies. L, lipid droplet; M, mitochondrion.

(C–E) Preembedding labeling of isolated mitochondria using anti-MOM22 antibodies (C), anti-MOM22N antibodies (D), and anti-MOM22C antibodies (E). In (E), intact mitochondria are not specifically labeled with the anti-MOM22C antibodies, yet occasionally small vesicles are labeled that are considered to represent inside-out vesicles of the outer mitochondrial membrane.

The bars represent 0.5 μm .

upon opening the intermembrane space with digitonin (Figure 4D), excluding the possibility that it possessed an endogenous protease resistance. The protected 12 kd fragment, which is resistant to extraction at alkaline pH (Figure 4A) or sonication (Figure 4B), thus represents the

C-terminal half of MOM22 including the membrane anchor sequence.

We conclude that MOM22 is anchored in the outer membrane by a single hydrophobic sequence (residues 85–105). The highly negatively charged N-terminal half is obvi-

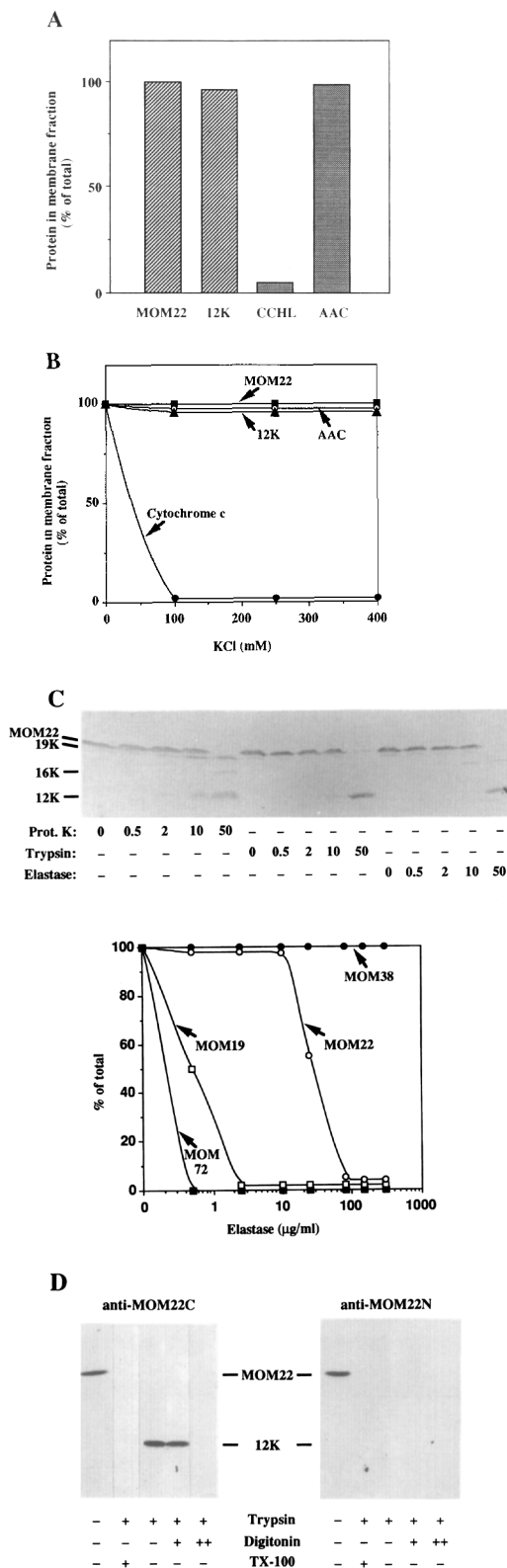


Figure 4. MOM22 Is an Integral Outer Membrane Protein with a Cytosolic Domain and a Domain in the Intermembrane Space
(A) MOM22 is not extracted from the membranes at pH 11.5. Mitochondria (100 µg of protein) were incubated in 100 mM Na₂CO₃ for 30 min at 0°C. Separation of pellets and supernatants was performed as described (Hartl et al., 1986). Analysis was by immunodecoration with

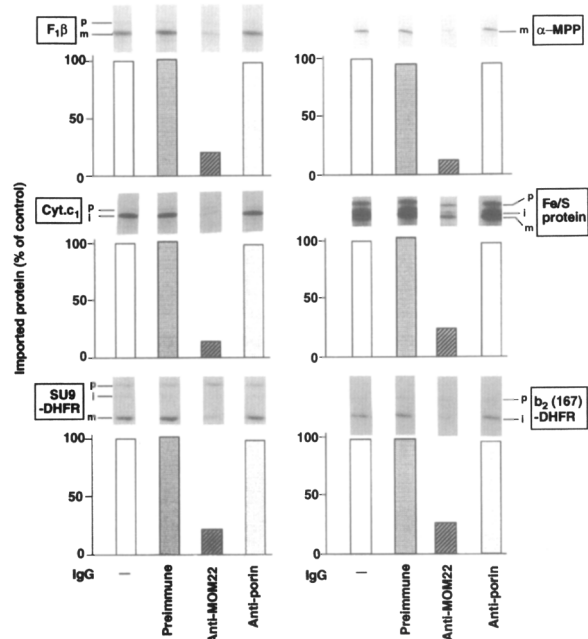


Figure 5. Antibodies against the N-Terminal Domain of MOM22 Inhibit the Import of Preproteins with Cleavable Targeting Sequences
IgGs (100 µg) from preimmune serum, antiserum directed against the N-terminal half of MOM22 (anti-MOM22), or antiserum directed against porin were prebound to isolated mitochondria (12.5 µg of protein per lane) (see Experimental Procedures). Mitochondrial preproteins were synthesized in rabbit reticulocyte lysates in the presence of [³⁵S]methionine and incubated with the energized mitochondria for 7 min at 25°C. Then, a treatment with trypsin (100 µg/ml) was performed for 20 min at 0°C. The mitochondria were reisolated and analyzed by SDS-PAGE, fluorography, and laser densitometry. The amount of protein imported in a control sample without IgGs was set to 100%. F₁β, F₁-ATPase subunit β; α-MPP, α subunit of the matrix-processing peptidase; Cyt. c₁, cytochrome c₁; Fe/S protein, Fe/S protein of the bc₁ complex; Su9-DHFR, fusion protein between the presequence of F₀-ATPase subunit 9 and DHFR; b₂(167)-DHFR, fusion protein between the N-terminal 167 amino acid residues of cytochrome b₂ and DHFR; p, i, and m, precursor, intermediate, or mature form of a protein, respectively.

antibodies against MOM22 (12K, a 12 kd fragment of MOM22), cytochrome c heme lyase (CCHL; an intermembrane space protein), and the AAC. The total amount of protein in pellet and supernatant was set to 100% (control).
(B) MOM22 is not released from the membranes by salt and sonication. Mitochondria were sonicated at various salt concentrations (Söllner et al., 1989, 1990). Membranes and supernatants were separated by centrifugation for 60 min at 166,000 × g and analyzed as described above. Cytochrome c, protein of the intermembrane space.
(C) Protease accessibility of MOM22. Mitochondria (50 µg of protein per lane) were incubated with proteinase K (Prot. K), trypsin, or elastase as indicated and analyzed with anti-MOM22C antibodies (upper and lower panels) or antibodies directed against MOM19, MOM38, or MOM72 (lower panel). 19K, 16K, and 12K, fragments of MOM22 of 19 kd, 16 kd, or 12 kd, respectively.
(D) The 12 kd (12K) fragment of MOM22 is recognized by anti-MOM22C, but not by anti-MOM22N antibodies. Mitochondria (75 µg of protein per lane) were incubated with 1% Triton X-100 or digitonin (plus sign, 0.05%; double plus sign, 0.15%; at 0.15%, the intermembrane space is opened [Hartl et al., 1986]) for 3 min at 0°C; parallel samples were left untreated. Where indicated, a treatment with trypsin (50 µg/ml) was performed. Analysis was performed by Western blotting with anti-MOM22N or anti-MOM22C antibodies.

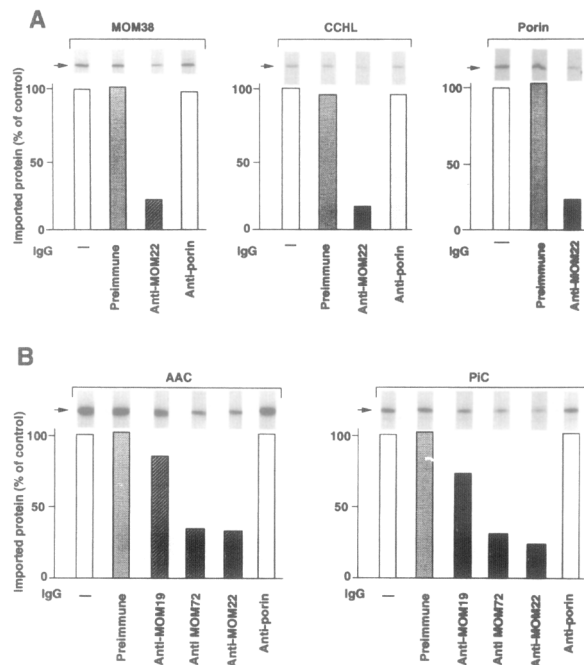


Figure 6. Antibodies against MOM22 inhibit the import of noncleavable preproteins

(A) Anti-MOM22 antibodies inhibit the import of noncleavable preproteins of the outer membrane (MOM38 and porin) and the intermembrane space (cytochrome c heme lyase [CCHL]). The experiments were performed as described in the legend of Figure 5 except that the import incubation for cytochrome c heme lyase was for 15 min at 0°C.

(B) Anti-MOM22 antibodies inhibit the import of noncleavable preproteins of the inner membrane (AAC and phosphate carrier [PIC]). The experiments were performed as described in the legend of Figure 5 with the following modifications. In additional samples, the inhibitory effects of antibodies against MOM19 or MOM72 were shown for comparison. After the import reaction, a treatment with proteinase K (200 µg/ml for AAC; 250 µg/ml for phosphate carrier) was performed. Quantitation was performed as described (Söllner et al., 1990). The arrows point toward the protein band resolved by SDS-PAGE.

ously exposed on the mitochondrial surface, while the C-terminal region is located on the intermembrane space side.

MOM22 Is Required for Import of Preproteins along Both Receptor Pathways

To study the function of MOM22, we obtained an antiserum against its surface-exposed domain. A fusion protein between the N-terminal 84 amino acid residues and the maltose-binding protein was expressed in *Escherichia coli* and used to raise antibodies in rabbits. The antiserum was monospecific for MOM22 (see Figure 2A, lane 3). We prepared immunoglobulins G (IgGs) and preincubated isolated mitochondria with the IgGs. Mitochondrial preproteins were synthesized in rabbit reticulocyte lysates in the presence of [³⁵S]methionine and imported into the isolated energized mitochondria (Figure 5). A series of preproteins with cleavable presequences were tested, including the precursors of matrix proteins (F₁-ATPase subunit β, mitochondrial processing peptidase subunit α, and a fusion

protein between the presequence of F₀-ATPase subunit 9 and dihydrofolate reductase [DHFR]), inner membrane proteins (cytochrome c₁ and the Fe/S protein of the bc₁ complex), and intermembrane space proteins (the fusion protein between the 167 N-terminal amino acid residues of cytochrome b₂ and DHFR). Control antibodies directed against the major outer membrane protein porin or from preimmune serum had no effect (Figure 5). Furthermore, we analyzed the import of several noncleavable preproteins, such as porin and MOM38 of the outer membrane and cytochrome c heme lyase of the intermembrane space (Figure 6A). The anti-MOM22 antibodies also strongly inhibited the import of these precursors.

All the preproteins tested use MOM19 as their main receptor, and the strong inhibitory effect of the anti-MOM22 antibodies was almost identical to that of anti-MOM19 antibodies (Söllner et al., 1989; Steger et al., 1990). We therefore asked whether anti-MOM22 antibodies would also interfere with import of preproteins that preferentially use MOM72 as an import receptor and are inhibited by anti-MOM19 antibodies only to a minor degree (Söllner et al., 1990; Steger et al., 1990). To this end, the import of the precursors of the AAC and the phosphate carrier of the inner membrane were studied. The anti-MOM22 antibodies also strongly inhibited the import of these two precursors (Figure 6B). This inhibition was far higher than that caused by anti-MOM19 antibodies (Figure 6B).

In summary, anti-MOM22 antibodies inhibit the import of both preproteins using MOM19 and preproteins using MOM72 as surface receptors. To exclude the possibility that the observed inhibition was an artifact caused by the divalent nature of the IgGs, we prepared Fab fragments from the anti-MOM22 antibodies and tested their effect on the import of various preproteins (Figure 7). The import of the preproteins was inhibited by the Fab fragments to about the same degree as by the IgGs. As control, we show that Fab fragments against porin had no inhibitory effect.

MOM22 Acts in the Transfer of Preproteins from Receptors to the GIP

At which stage of the import pathway is MOM22 required? The transport of the AAC across the outer membrane can be divided into two steps (Pfanner et al., 1987b): binding

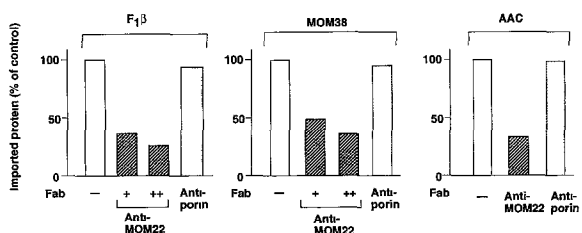


Figure 7. Fab fragments directed against MOM22 inhibit protein import

The experiments were performed as described in the legend to Figure 5 except that Fab fragments (40 µg [plus sign] or 80 µg [double plus sign] and the other samples) were used instead of IgGs.

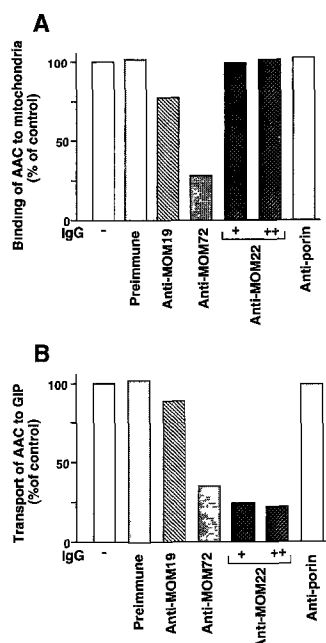


Figure 8. Antibodies against MOM22 Do Not Inhibit Binding to the Receptors, but Inhibit the Insertion of the AAC into the GIP

(A) Binding of the AAC to the surface receptors is not inhibited by anti-MOM22 antibodies. Mitochondria (12.5 μ g of protein) were pre-treated with apyrase to deplete ATP and incubated with IgGs as indicated (60 μ g [plus sign] or 120 μ g [double plus sign and the other samples]) and described in Experimental Procedures. Then, the membrane potential was dissipated, and the precursor of the AAC was bound to the mitochondria. Analysis was performed as described in the legend of Figure 5.

(B) Insertion of the AAC into the GIP is inhibited by anti-MOM22 antibodies. Isolated mitochondria were preincubated with IgGs as described above. Transport of the precursor of the AAC to the GIP was performed in the presence of ATP (no apyrase) as well as in the absence of a membrane potential, followed by a treatment with 20 μ g/ml proteinase K as described in Experimental Procedures.

to the surface receptors MOM72 and MOM19 (Söllner et al., 1990, 1992; Steger et al., 1990) and insertion into the outer membrane at the GIP (Pfaller et al., 1988; Söllner et al., 1992). In Figure 8A, accumulation of the precursor of the AAC at the surface receptors was analyzed. Preincubation of mitochondria with antibodies against MOM72 or MOM19 inhibited this binding step; with anti-MOM72 antibodies, the inhibition was about 70%, and with anti-MOM19 antibodies it was 15%–20% (Figure 8A), in agreement with the relative contributions of both receptors to the import of the AAC (Steger et al., 1990). Antibodies against MOM22, however, did not show any inhibitory effect on binding (Figure 8A). We conclude that MOM22 is involved in a transport step occurring after the initial binding of precursors to their receptors. We then tested the transport of the AAC into the GIP. Anti-MOM22 antibodies had a strong inhibitory effect on the formation of the GIP intermediate (Figure 8B); the degree of inhibition of this step was similar to that seen with the overall import of the AAC (see Figure 6B).

A similar stage dependence of import inhibition by anti-MOM22 antibodies was also observed for the precursor

of the phosphate carrier. All together, these data suggest that MOM22 is required for insertion of preproteins into the GIP.

Discussion

We have characterized MOM22, a component of the mitochondrial receptor complex, and have provided evidence that it is a functional linker between the mitochondrial surface receptors and the GIP in the outer membrane. MOM22, with its 154 amino acid residues, can be structurally divided into three parts: the N-terminal half on the cytosolic side of the outer membrane, an uncharged transmembrane segment, and the hydrophilic C-terminal domain on the intermembrane space side. MOM22 thus has an inverted orientation in comparison with that of MOM19 or MOM72, which exposes the C-terminus to the cytosol. The most prominent characteristic of MOM22 is the presence of a cluster of 18 negative charges (with no positive charge) in the cytosolic domain.

Antibodies directed against MOM22 inhibited the import of all preproteins analyzed that use the mitochondrial receptor complex. This is in contrast with antibodies directed against the receptors MOM19 or MOM72, which showed different inhibitory effects on different subclasses of preproteins. It may be argued that the antibodies against MOM22 unspecifically block the surface of the mitochondrial receptor complex. This can be excluded on the basis of the following observations. First, Fab fragments were found to exert the same inhibitory effect as divalent antibodies; second, the anti-MOM22 antibodies did not interfere with the binding of the preproteins to their receptors.

It is the subsequent step, the transfer of preproteins into the GIP, that requires MOM22. The import pathways via both receptors MOM19 and MOM72 appear to converge at MOM22. Here the preprotein with its positively charged signal sequence must insert into the translocation machinery of the outer membrane. We propose that the cytosolic domain of MOM22 facilitates the entry of preproteins into the translocation pore, in cooperation with receptors and components of the GIP.

Mitochondrial targeting signals have the potential to form amphipathic α helices in which the positively charged amino acid residues and the hydrophobic residues are facing opposite sites of the helix (Roise et al., 1986; von Heijne, 1986). It is tempting to speculate that the cytosolic domain of MOM22, with its abundant negative charges, provides a surface for the transient binding of the positively charged aspect of the signal sequences. After the initial recognition by the receptor MOM19 or MOM72, the signal sequences would be presented to MOM22. One may further speculate that the conformation of the signal acquired upon this interaction with MOM22 renders the preprotein competent for insertion into the translocation pore.

Translocation of preproteins across membranes has also been characterized in considerable detail with the endoplasmic reticulum and the plasma membrane of *E. coli*, and quite a number of components of the transport machineries have been identified and sequenced (reviewed in Wickner et al., 1991; Rapoport, 1992; Sanders

and Schekman, 1992). The signal/leader sequences of preproteins translocated into the endoplasmic reticulum or out of *E. coli* are quite different from the signal sequences of mitochondrial preproteins. They are characterized by a hydrophobic core usually preceded by a single positive charge. Thus, a highly negatively charged surface for facilitating insertion into a translocation pore may not be needed. In fact, of the components of the endoplasmic reticulum or the *E. coli* transport machineries identified so far, none has a domain that is as negatively charged as the N-terminus of MOM22. However, a situation comparable to that in the mitochondrial outer membrane may exist in the chloroplast envelope. Transit sequences of chloroplast preproteins resemble mitochondrial signal sequences in the prevalence of positive charges (Keegstra and von Heijne, 1992). It will be interesting to see whether a component similar to or equivalent to MOM22 exists in the chloroplast outer envelope membrane.

Experimental Procedures

Isolation of Mitochondria

N. crassa wild-type 74A was grown and harvested as described (Schleyer et al., 1982). For labeling with ^{35}S , 5 mCi [^{35}S]sulfate (Amersham) was added per liter of culture (growth medium containing 0.08 mM unlabeled sulfate). Mitochondria were isolated as described (Schleyer et al., 1982) and resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH [pH 7.2]) at a final protein concentration of 5 mg/ml.

Isolation and Sequencing of MOM22 cDNA

Antibody screening was performed with a size-fractionated λ gt11 library of *N. crassa* cDNA (Young and Davis, 1983; Schneider et al., 1990). The resulting cDNA clones were subcloned into the EcoRI site of pGEM4 (Promega Biotec; Melton et al., 1984; Söllner et al., 1990) and pUX (Bressan and Stanley, 1987). Supercoil sequencing was performed with denatured plasmids (Chen and Seeburg, 1985) according to the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (a modified T7 DNA polymerase, U. S. Biochemical; Tabor and Richardson, 1987) and ^{35}S -labeled dATP (Amersham). To isolate a full-length cDNA clone, the λ gt11 library of *N. crassa* cDNA was screened using the radiolabeled cDNA insert (Rigby et al., 1977) identified from the first screen. The full-length cDNA was subcloned into pGEM4 and sequenced using MOM22-specific cDNA primers and subcloned restriction fragments of the cDNA.

Expression of the N-Terminal Domain of MOM22 in *E. coli*

Using two MOM22-specific cDNA primers, one for the coding strand including the start ATG (5'-GCG AAT TCC ATG GTT CAG CTT ACC GAG-3', introducing a NcoI site at the ATG), the other for the complementary strand in the reverse orientation (3'-GCG GCC GCT TCC TCT CAC-5'), the cDNA from nucleotides 191–441 (coding for the first 84 amino acid residues of MOM22) was amplified by polymerase chain reaction (Saiki et al., 1988; Innis et al., 1988). The resulting polymerase chain reaction fragment was digested with NcoI and ligated to an NcoI-EcoRI-linearized maltose–fusion protein vector, pMALcRI (New England Biolabs; Maina et al., 1988). The resulting linearized fragment was treated with the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim; Sambrook et al., 1989) for 15 min at 37°C in the presence of 100 μM deoxynucleotides (Boehringer Mannheim) and Klenow buffer (10 mM MgSO_4 , 1 mM dithiothreitol, 50 mM Tris-HCl [pH 7.2]), ligated again, and transformed (Hanahan, 1983) into the *E. coli* strain TB1 (New England Biolabs).

Expression of the resulting fusion protein between the N-terminal half of MOM22 and the maltose-binding protein was induced for 2 hr at 37°C with 1 mM isopropyl β -D-thiogalactopyranoside (Boehringer Ingelheim). The cells were harvested by centrifugation, incubated with water-solved lysozyme and DNAase (4 mg/ml and 1 mg/ml, respec-

tively; Serva-Boehringer Mannheim) for 10 min at 0°C, frozen and thawed several times, and mixed with 2-fold sample buffer (120 mM Tris-HCl [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 0.04% [w/v] bromophenol blue, 2% [v/v] 2-mercaptoethanol) (Sambrook et al., 1989). After SDS-PAGE, the proteins were transferred to nitrocellulose (semidry electrophoretic transfer for 1–2 hr at 200 mA; Kyshe-Andersen, 1984) in blot buffer (20 mM Tris, 150 mM glycine, 0.02% SDS, and 20% methanol). The band reacting with anti-MOM22 antibody was excised and used for generation of antibodies.

Raising of Antisera, Preparation of IgGs and Fab Fragments, and Immunoprecipitation

Antisera were raised in rabbits against chemically synthesized oligopeptides that were coupled to keyhole limpet hemocyanin (Harlow and Lane, 1988) or against protein expressed in *E. coli*. The complement was heat inactivated (20 min at 56°C), and IgGs were prepared using protein A–Superose column chromatography (Pharmacia). Bound IgGs were eluted with 0.1 M citrate (pH 3.0), neutralized with 2 M Tris base (pH 8.8), dialysed against water, lyophilized, and dissolved in SEM buffer at a protein concentration of 5–50 mg/ml. Aliquots were frozen at –20°C.

IgGs were digested with papain according to Mage (1981). Fab fragments were separated from IgGs and Fc fragments by using a protein A–Superose column. Fab fragments were dialysed against water, lyophilized, and dissolved in SEM buffer at a protein concentration of 5–50 mg/ml. Aliquots were frozen at –20°C.

For immunoprecipitation of the receptor complex, mitochondria (1 mg/ml protein) were lysed in digitonin buffer (0.5% [w/v] digitonin, 3% [w/v] bovine serum albumin [BSA], 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride in SEM buffer) for 15 min at 0°C, followed by centrifugation for 15 min at 25,000 \times g. This extract was incubated with protein A–Sepharose carrying specific antibodies in digitonin buffer for 90 min at 0°C. The immunoprecipitates were washed in digitonin buffer (without BSA). Dissociation of the immunoprecipitates was performed by adding sample buffer, followed by SDS-PAGE and fluorography.

Mitochondrial Binding and Import of Preproteins In Vitro

Specific messenger RNA transcripts coding for preproteins were synthesized with SP6 polymerase (Melton et al., 1984) from pGEM4 plasmids and translated in rabbit reticulocyte lysate (Pelham and Jackson, 1976) in the presence of [^{35}S]methionine (Amersham). Postribosomal supernatants were prepared and supplemented as described (Zimmermann and Neupert, 1980).

IgGs or Fab fragments were incubated with isolated mitochondria (12.5 μg of protein per lane) in SEM or BSA buffer (250 mM sucrose, 3% [w/v] BSA, 80 mM KCl, 5 mM MgCl_2 , 10 mM MOPS-KOH [pH 7.2]) for 35 min at 4°C in a final volume of 50 μl . Import reactions contained, in addition to the pretreated mitochondria, 8 mM potassium ascorbate and 0.2 mM tetramethylphenylenediamine, 10 mM unlabeled methionine, 1–10 μl of reticulocyte lysate, and BSA buffer up to a final volume of 200 μl . All reactions were made chemically identical by adding the same volume of reagent-free solvent to the control samples. Incubation was usually performed for 7 min at 25°C, except for cytochrome c heme lyase (15 min at 0°C). The analysis was thereby performed in the linear range of the import kinetics of the respective precursors (Pfanner et al., 1987a; Söllner et al., 1989, 1990; Lill et al., 1992).

For binding of the AAC to the mitochondrial surface, reticulocyte lysate and mitochondria were separately pretreated with apyrase (5 U/ml; Sigma grade VIII; Pfanner and Neupert, 1986) for 15 min at 25°C or 25 min at 4°C, respectively. The binding reaction was performed in the presence of antimycin A (8 μM), oligomycin (20 μM), and valinomycin (0.5 μM) (Sigma; Pfanner et al., 1987b; Pfaller et al., 1988) to dissipate the membrane potential. The insertion of the AAC into the outer membrane (the GIP) was performed in the absence of a membrane potential but in the presence of ATP (no apyrase treatment) (Pfanner and Neupert, 1987). The mitochondria were reisolated, washed with SEM buffer, and treated with proteinase K for 20 min at 0°C (200 $\mu\text{g}/\text{ml}$ for imported AAC; 20 $\mu\text{g}/\text{ml}$ for AAC at the GIP; mock treatment for AAC at the receptor stage) (Söllner et al., 1991). The mitochondria were reisolated and subjected to SDS-PAGE, fluorography, and laser densitometry (Söllner et al., 1991).

Protease Treatment of Mitochondria and Western Blotting

Mitochondria (1 mg/ml protein) were treated with protease (added from a stock solution of 2.5 mg/ml in SEM buffer). After 20 min at 0°C, the protease was inactivated by addition of 1 mM phenylmethylsulfonyl fluoride and, in the case of trypsin, a 20-fold weight excess of soybean trypsin inhibitor and incubated for 10 min at 0°C (15 min at 25°C in the case of elastase). Mitochondria were then reisolated, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose (semidry electrophoretic transfer). The nitrocellulose was incubated in 5% milk powder and Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris [pH 7.2]) for 20 min at 25°C. Then, the first antiserum was added (dilution 1:1000 in TBS-5% milk powder; 1.5 hr at 25°C). After extensive washing in TBS and TBS-0.1% Triton X-100, the blot was incubated with an enzyme-coupled antibody directed against rabbit IgGs. Two methods were applied: horseradish peroxidase or alkaline phosphatase (both 1:1000 in TBS-5% milk powder; 1 hr at 25°C). After extensive washing in TBS and TBS-0.1% Triton X-100, color reaction was performed for alkaline phosphatase with bromochlorodolyl phosphate and nitro blue tetrazolium in 100 mM NaCl, 5 mM MgCl₂, 100 mM Tris-HCl (pH 9.5) for 1-5 min at 25°C; in the case of horseradish peroxidase, the reaction was performed either with diaminobenzidine and H₂O₂ (0.1%) in 50 mM Tris-HCl (pH 7.5) or with the ECL Western Blotting System (Amersham).

Electron Microscopy

For immunocytochemistry, hyphae were fixed in 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 60 min at 0°C, dehydrated in a graded ethanol series, and embedded in Lowidryl K₄M. Immunolabeling was performed on ultrathin Lowicryl sections using monospecific antibodies against MOM22 and gold-conjugated goat anti-rabbit antibodies according to the instructions of the manufacturer (Amersham).

For preembedding labeling, 50 µl of antiserum, 50 µl of isolated mitochondria (1 mg/ml protein in SEM buffer), and 100 µl of buffer A (8 µl of a 10-fold concentrated phosphate-buffered saline-glycine stock solution in 92 µl of SEM buffer [final concentration of glycine, 8 mM]) were mixed. Upon incubation for 1 hr at 0°C, the organelles were reisolated, washed twice in buffer A, and resuspended in 500 µl of buffer A containing gold-conjugated goat anti-rabbit antibodies (dilution 1:10). After incubation for 1 hr at 0°C, the organelles were again reisolated, washed twice in buffer A, and fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 30 min at 0°C. Postfixation was in a mixture of 0.5% (w/v) OsO₄ and 2.5% (w/v) K₂Cr₂O₇ in the same cacodylate buffer for 1 hr at room temperature. The samples were posthydrated in 1% (w/v) aqueous uranyl acetate for 8-16 hr, dehydrated in a graded ethanol series, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

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